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Journal

Biomacromolecules, 21(1)

ISSN

1525-7797

Authors

Perlin, Pesach
Scott, Wendell A
Deming, Timothy J

Publication Date

2020

DOI

10.1021/acs.biomac.9b01065

Peer reviewed

Modification of poly(5,6-epoxy-L-norleucine) gives functional polypeptides with alternative side-chain linkages

Pesach Perlin[a], Wendell A. Scott[a] and Timothy J. Deming[a,b]*

[a] Department of Chemistry and Biochemistry, University of California, Los Angeles, CA 90095

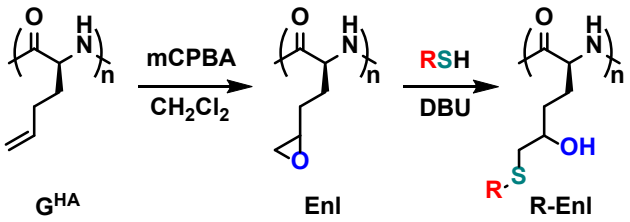
[b] Department of Bioengineering, University of California, Los Angeles, CA 90095

Abstract The preparation and characterization of a new epoxide containing polypeptide, poly(5,6-epoxy-L-norleucine), via post-polymerization modification of poly(L-homoallylglycine) is described. Addition of thiols to the epoxide groups in poly(5,6-epoxy-L-norleucine) was studied as a means to prepare side-chain functional polypeptides. The solution properties of the derivatized polypeptides were studied in water, and compared to similar thioether containing functional polypeptides prepared via different routes. Subtle differences in side-chain linkage chemistry were found to influence polypeptide solubility, chain conformation in solution, and thermoresponsive behavior. Poly(5,6-epoxy-L-norleucine) was found to be useful as a readily prepared intermediate that can be reacted with thiols to give a variety of functional polypeptides.

Introduction

Side-chain functional polypeptides have generated much interest in recent years due to their ability to present biologically relevant functional groups, and since many functional groups provide a means to obtain properties that are difficult to realize through use of canonical amino acids.[1,2] A variety of strategies have emerged for synthesis of functionalized polypeptides, with considerable effort focused on post-

polymerization conjugation reactions utilizing functional side-chain groups such as azides,[3,4] alkynes,[5-7] alkenes,[8-11] oxyamines,[12] thiols,[13] and thioethers.[14] On the contrary, epoxides have been used extensively for conjugation reactions as side-chain groups in synthetic polymers,[15,16] but not in polypeptides. Epoxides are readily prepared by oxidation of alkenes, which have been incorporated into a variety of polypeptides,[1,8-11] but only one example of an epoxide side-chain functionalized polypeptide has been reported.[8] In this example, the epoxide groups were used only for chain crosslinking, and not for conjugation reactions.[8]

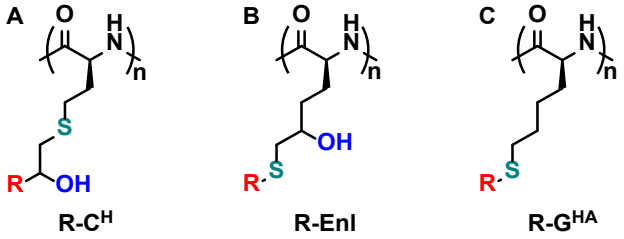


Scheme 1. Oxidation of poly(L-homoallylglycine), $\mathbf{G^{HA}}$, to give poly(5,6-epoxy-L-norleucine), **Enl**, for synthesis of thiol functionalized polypeptides, **R-Enl**.

Our group recently reported the synthesis of poly(L-homoallylglycine), $\mathbf{G^{HA}}$, a readily prepared soluble, α-helical polypeptide that is converted to functional derivatives via thiol-ene reactions with thiols.[11] We recognized that $\mathbf{G^{HA}}$ could potentially be oxidized to give the derivative poly(5,6-epoxy-L-norleucine), **Enl**, which would also be amenable toward functionalization with thiols (Scheme 1). If successful, not only would **Enl** be useful for formation of functional conjugates with thiols, but it would also allow preparation of side-chain structural analogs of thioether containing polypeptides obtained via other routes.[11,17] This feature would allow comparison of polypeptides containing similar functional groups, but with small structural differences in side-chain linkages, providing insights on how linkage structure influences properties such as polypeptide solubility and chain conformation.

Here, we sought to prepare **Enl** from $\mathbf{G^{HA}}$, and study its utility for formation of conjugates with functional thiols, **R-Enl**, as outlined in Scheme 1. Specifically, functional thiols were chosen to favor formation of water soluble polypeptide derivatives, which are potentially useful for downstream

biological studies and for analysis of chain conformations in aqueous media.[1] Water soluble **R-Enl** derivatives were also selected since they could be compared to previously reported water soluble thioether containing polypeptides prepared via other routes, including thiol-ene conjugates of the parent $\mathbf{G^{HA}}$ chains, $\mathbf{R\text{-}G^{HA}}$ (Scheme 2, see Supporting Information (SI) Scheme S1).[11,17] Upon conjugation of an identical thiol, the only difference between **R-Enl** and $\mathbf{R\text{-}G^{HA}}$ products is the presence of additional alcohol groups in the side chains of **R-Enl**.



Scheme 2. Structural variants of functional polypeptides containing thioether side-chain groups. A) Poly(S-alkyl-L-homocysteine), $\mathbf{R\text{-}C^{H}}$, synthesized via epoxide alkylation and demethylation of poly(L-methionine).[17] B) Thiol modified poly(5,6-epoxy-L-norleucine), **R-Enl**, via nucleophilic ring-opening. C) Thiol modified poly(L-homoallylglycine), $\mathbf{R\text{-}G^{HA}}$, via thiol-ene conjugation.[11] See Scheme S1 for details on preparation of $\mathbf{R\text{-}C^{H}}$ and $\mathbf{R\text{-}G^{HA}}$.

R-Enl polypeptides are also structural isomers of poly(S-alkyl-L-homocysteines), $\mathbf{R\text{-}C^{H}}$, which are readily prepared from poly(L-methionine) via alkylation with epoxides followed by demethylation of the sulfonium intermediates (Scheme 2, see Scheme S1).[17] Thioether containing polypeptides have been found to possess many useful properties, such as reversible temperature dependent solubility in water,[17] and reversible α-helix to coil chain conformational switching via oxidation or alkylation under mild conditions in water.[18] Thus, we sought to prepare **R-Enl** polypeptides since this would not only provide a new method for synthesis of functional polypeptides, but also would provide side-chain variants of thioether containing polypeptides and potentially provide insights on how subtle structural differences affect polypeptide solution properties.

Experimental Section

Materials and Instrumentation Unless otherwise specified, all post-polymerization modification chemistry was performed in glass vials under ambient atmosphere. Reactions of **Enl$_{55}$** with thiols were performed in 1 dram screw top glass vials capped with septa to allow sparging. Small molecule chemistry was performed in heat-dried glassware under a nitrogen atmosphere, unless otherwise specified. THF and hexanes were each degassed with dinitrogen and passed through an alumina column before use. All other reagents and solvents were used as received. Poly(L-homoallylglycine)$_{55}$ (**G$^{HA}_{55}$**), oligoethylene glycol-thiols (except for 2-(2-methoxyethoxy)ethane-1-thiol and 2-(2-ethoxyethoxy)ethane-1-thiol), and *N,N*-dimethyl-cysteamine-carboxybetaine have been previously reported and were synthesized using literature procedures.[11,19] Unless otherwise specified, all reactions were performed at ambient temperature (*ca*. 20 °C). In-house deionized water was used for all aqueous chemistry and dialysis unless otherwise specified. Thin-layer chromatography was performed with EMD gel 60 F254 plates (0.25 mm thickness) and spots were visualized using a UV lamp or $KMnO_4$ stain. Silicycle Siliaflash G60 silica (60-200 μm) was used for all column chromatography. Silica used for chromatographic purification of NCA monomers was dried under vacuum at 250 °C for 48 hours and then stored in a dinitrogen filled glovebox. Compositions of mobile phases used for chromatography are given in volume percent. Dialysis was performed with regenerated cellulose tubing obtained from Spectrum labs. NMR spectra of solution samples were recorded on a Bruker AV400 instrument with chemical shifts reported relative to the deuterated solvent used. Solid state ^{13}C Cross Polarization-Magic Angle Spinning NMR was conducted on a Bruker AV III HD instrument with a magnetic field of 14.1 T. 10 mg of **G$^{HA}_{55}$**, 10 mg of **Enl$_{55}$**, and 50 mg of **GL-Enl$_{55}$** were used for acquisition of spectra. The contact time was 1.5 ms with a recycle delay of 5 s. The sample spinning rate was 10 kHz. Samples for circular dichroism (CD) spectroscopy were prepared using deionized water filtered through a Millipore Milli-Q Biocel A10 filter system unless otherwise specified.

CD spectra were collected using 0.1 mg /mL solutions of polypeptide on either an Olis DSM 10 spectrophotometer or a JASCO J-715 spectrophotometer using a 0.1 cm path length quartz cuvette. Percent α-helical content of polypeptides was estimated using the formula: % α-helix = 100 × (−$[\theta]_{222}$ + 3000)/39000), where $[\theta]_{222}$ is the measured molar ellipticity at 222 nm in (deg×cm^2/dmol).[20] Solid state Fourier transform infrared (FTIR) spectra were collected using a Thermo Scientific Nicolet iS5 FTIR spectrophotometer with an ID7 ATR Sampling Accessory. Tandem gel permeation chromatography/light scattering (GPC/LS) was performed at 25 ºC using an SSI Accuflow Series III pump equipped with Wyatt DAWN EOS light scattering and Optilab REX refractive index detectors. Separations were achieved using 100 Å and 1000 Å PSS-PFG 7 μm columns at 30 ºC with 0.5% (w/w) KTFA in HFIP as eluent and sample concentrations of 10 mg/ml. Cloud point temperature measurements were recorded at a wavelength of 500 nm on an HP 8453 spectrophotometer equipped with an Agilent 8909A temperature controller.

poly(5,6-epoxy-L-norleucine)$_{55}$, ***Enl$_{55}$*** A sample of **G^{HA}_{55}** (45.2 mg) was dissolved in DCM (5 mg/mL). mCPBA was added to the solution (210 mg, 2.5 eq per each alkene group). The reaction was let stir for 2 days then transferred to a 2000 MWCO dialysis bag. The reaction mixture was dialyzed against methanol for 1 day, then DI water for 1 day, with 2 dialysate changes daily. The dialyzed polymer was lyophilized to dryness, yielding the product as a white solid (41.5 mg, 81% yield). ^{1}H NMR (400 MHz, DMSO-d_6, 25 °C): δ 8.68-7.50 (br m, 1H), 4.36-3.85 (br m, 1H), 3.03-2.78 (br m, 1H), 2.70-2.56 (br m, 1H), 2.42-2.28 (br m, 1H), 2.20-1.26 (br m, 4H) ^{13}C NMR (CP-MAS, 25 °C): δ 175.8, 57.3, 50.9, 46.2, 26.9.

General procedure for reactions of epoxide groups in ***Enl$_{55}$*** *with thiols* In a 1 dram screw top vial, a sample of **Enl$_{55}$** was dissolved in DMF to give a 3 mg/mL solution. 5 equivalents of thiol were added per each epoxide group and N_2 was bubbled through the solution for 10 minutes. 6 equivalents of DBU (50 mg/mL in DMF, purged with N_2) were added to the reaction mixture via syringe and the reaction was let stir under nitrogen at 40 °C for 2 days. The reaction mixture was then dialyzed against DI water

(2000 MWCO) for 2 days with 2 water changes daily. The dialyzed polypeptide solution was lyophilized to dryness, yielding the product a white solid.

2-methoxyethane-1-thiol modified poly(5,6-epoxy-L-norleucine), ***mEG$_1$-Enl$_{55}$*** The general procedure for reactions of epoxide groups in **Enl$_{55}$** with thiols was followed. **Enl$_{55}$** (5.0 mg) and 2-methoxyethanethiol were used to prepare the product, obtained as a white solid (8.5 mg, 100% yield). ^{1}H NMR (400 MHz, d-TFA, 25 °C): δ 5.04-4.38 (br m, 1H), 4.20-3.98 (br m, 1H), 3.91 (br t, J = 5.9 Hz, 2H), 3.68-3.51 (br m, 3H), 3.15-2.60 (br m, 4H), 2.32-1.76 (br m, 4H).

2-(2-methoxyethoxy)ethane-1-thiol modified poly(5,6-epoxy-L-norleucine), ***mEG$_2$-Enl$_{55}$*** The general procedure for reactions of epoxide groups in **Enl$_{55}$** with thiols was followed. **Enl$_{55}$** (2.5 mg) and 2-(2-methoxyethoxy)ethanethiol were used to prepare the product, obtained as a white solid (5.0 mg, 98% yield). ^{1}H NMR (400 MHz, D_2O, 25 °C): δ 4.27-3.87 (br m, 1H), 3.79-3.43 (br m, 7H), 3.27 (s, 3H), 2.80-2.44 (br m, 4H), 2.15-1.26 (br m, 4H)

2-(2-ethoxyethoxy)ethane-1-thiol modified poly(5,6-epoxy-L-norleucine), ***eEG$_2$-Enl$_{55}$*** The general procedure for reactions of epoxide groups in **Enl$_{55}$** with thiols was followed. **Enl$_{55}$** (6.3 mg) and 2-(2-ethoxyethoxy)ethanethiol were used to prepare the product, obtained as a white solid (9.9 mg, 73% yield). ^{1}H NMR (400 MHz, d-TFA, 25 °C): δ 5.10-4.46 (br m, 1H), 4.21-3.69 (br m, 9H), 3.37-2.58 (br m, 4H), 2.43-1.76 (br m, 4H), 1.41 (t, J = 7.1, 3H).

2-[2-(2-methoxyethoxy)ethoxy]ethanethiol modified poly(5,6-epoxy-L-norleucine), ***mEG$_3$-Enl$_{55}$*** The general procedure for reactions of epoxide groups in **Enl$_{55}$** with thiols was followed. **Enl$_{55}$** (2.6 mg) and 2-[2-(2-methoxyethoxy)ethoxy]ethanethiol were used to prepare the product, obtained as a white solid

(5.1 mg, 82% yield). ^{1}H NMR (400 MHz, D_2O, 25 °C): δ 4.32-3.87 (br m, 1H), 3.79-3.31 (br m, 11H), 3.30-3.20 (br s, 3H), 2.82-2.40 (br m, 4H), 2.12-1.33 (br m, 4H).

2-[2-[2-(2-methoxyethoxy)ethoxy]ethoxy]ethanethiol modified poly(5,6-epoxy-L-norleucine), ***mEG$_4$-Enl$_{55}$*** The general procedure for reactions of epoxide groups in **Enl$_{55}$** with thiols was followed. **Enl$_{55}$** (2.0 mg) and 2-[2-[2-(2-methoxyethoxy)ethoxy]ethoxy]ethanethiol were used to prepare the product, obtained as a white solid (5.5 mg, 100% yield). ^{1}H NMR (400 MHz, D_2O, 25 °C): δ 4.27-3.88 (br m, 1H), 3.79-3.47 (br m, 15H), 3.27 (s, 3H), 2.81-2.45 (br m, 4H), 2.11-1.32 (br m, 4H).

1-mercapto-11-hydroxy-3,6,9-trioxaundecane modified poly(5,6-epoxy-L-norleucine), ***EG$_4$-Enl$_{55}$*** The general procedure for reactions of epoxide groups in **Enl$_{55}$** with thiols was followed. **Enl$_{55}$** (2.0 mg) and 1-mercapto-11-hydroxy-3,6,9-trioxaundecane were used to prepare the product, obtained as a white solid (4.6 mg, 88% yield). ^{1}H NMR (400 MHz, D_2O, 25 °C): δ 4.38-3.82 (br m, 1H), 3.81-3.17 (br m, 15H), 2.94-2.26 (br m, 4H), 2.07-1.30 (br m, 4H).

1-thioglycerol modified poly(5,6-epoxy-L-norleucine), ***GL-Enl$_{55}$*** The general procedure for reactions of epoxide groups in **Enl$_{55}$** with thiols was followed. **Enl$_{55}$** (4.3 mg) and 1-thioglycerol were used to prepare the product, obtained as a white solid (6.0 mg, 75% yield). ^{1}H NMR (400 MHz, D_2O, 25 °C): δ 4.29-3.91 (br m, 1H), 3.81-3.62 (br m, 2H), 3.62-3.51 (br m, 1H), 3.50-3.42 (m, 1H), 2.82-2.28 (br m, 4H), 2.17-1.31 (br m 4H). ^{13}C NMR (CP-MAS, 25 °C): δ 176.2, 71.3, 65.4, 58.0, 33.4, 27.7.

N,N-dimethyl-cysteamine-carboxybetaine modified poly(5,6-epoxy-L-norleucine), ***CB-Enl$_{55}$*** **Enl$_{55}$ (**2.5 mg) and *N,N*-dimethyl-cysteamine-carboxybetaine (36 mg, 10 eq per each epoxide group) were added to a 1 dram screw cap vial. DI water (420 μL) was added and the vial was briefly placed in an

ultrasonication bath resulting in formation of a fine suspension. DBU was then added (40 μL, 10.5 eq) and the reaction was stirred vigorously for 48 hr to give a turbid suspension, which was transferred to a 2000 MWCO dialysis bag. The reaction mixture was dialyzed against DI water for 2 days with 2 water changes daily then lyophilized to dryness, yielding the product as a white solid (5.6 mg, 97% yield). ^{1}H NMR (400 MHz, 10% DCl in D_2O, 25 °C): δ 2.60-2.21 (br m, 3H), 2.00-1.68 (br m, 3H), 1.46-1.19 (br m, 6H), 1.12-0.95 (br m, 2H), 0.90-0.64 (br m, 2H), 0.02-⁻0.52 (br m, 4H).

2-(2-methoxyethoxy)ethane-1-thiol modified poly(5,6-epoxy-L-norleucine sulfoxide), ***mEG$_2$-Enl$^O_{55}$*** This compound was synthesized using a procedure for a related polypeptide.[11] **mEG$_2$-Enl$_{55}$** (7.5 mg) and CSA (1.3 mg) were dissolved in DI water (0.88 mL). An aqueous solution of TBHP (70 % (w/w), 16 eq per each thioether group) was then added. The reaction was let stir overnight. The reaction mixture was then transferred to a 2000 MWCO dialysis bag and dialyzed against DI water for 48 hours with 2 water changes daily. The dialyzed polymer was lyophilized to dryness to give the product as a white solid (6.6 mg, 83% yield). ^{1}H NMR (400 MHz, D_2O, 25 °C): 4.25-4.10 (br m, 1H), 4.06-3.95 (br m, 1H), 3.89-3.73 (br m, 2H), 3.64-3.47 (br m, 4H), 3.26 (s, 3H), 3.20-2.77 (br m, 4H), 2.09-1.44 (br m, 4H).

2-(2-ethoxyethoxy)ethane-1-thiol modified poly(5,6-epoxy-L-norleucine sulfoxide), ***eEG$_2$-Enl$^O_{55}$***
This compound was synthesized using a procedure for a related polypeptide.[11] **eEG$_2$-Enl$_{55}$** (7.4 mg) and CSA (1.2 mg) were dissolved in DI water (0.87 mL). An aqueous solution of TBHP (70 % (w/w), 16 eq per each thioether group) was then added. The reaction was let stir for 2 days. The reaction mixture was then transferred to a 2000 MWCO dialysis bag and dialyzed against DI water for 48 hours with 2 water changes daily. The dialyzed polymer was lyophilized to dryness to give the product as a white solid (7.1 mg, 91% yield). ^{1}H NMR (400 MHz, D_2O, 25 °C): 4.29-4.09 (br m, 1H), 4.07-3.95 (br m, 1H), 3.91-3.75

(br m, 2H), 3.65-3.52 (br m, 4H), 3.48 (q, J = 7.1, 2H), 3.22-2.77 (br m, 4H), 2.01-1.48 (br m, 4H), 1.08 (t, J = 7.0, 3H).

Results and Discussion

The conversion of $\mathbf{G^{HA}_{55}}$ to $\mathbf{Enl_{55}}$ was attempted using standard mCPBA oxidation conditions that have been successfully used to oxidize other alkene containing polymers[8] as well as small molecule derivatives of L-homoallylglycine (Scheme 1).[21,22] The product $\mathbf{Enl_{55}}$ was isolated and purified by dialysis against methanol, then DI water, and followed by lyophilization of the resulting polypeptide precipitate. $\mathbf{Enl_{55}}$ was found to be soluble in polar organic solvents, such as DMF, DMSO, and HFIP. The ^{1}H NMR spectrum of $\mathbf{Enl_{55}}$ showed nearly complete disappearance of alkene resonances, and the appearance of new resonances at 2.90, 2.63, and 2.35 ppm that are consistent with literature data for the epoxide group of 5,6-epoxy-L-norleucine (see SI).[8,21,22] Due to the broad linewidths observed in ^{1}H NMR, solid-state ^{13}C CP-MAS NMR and FTIR were also used as more sensitive techniques to follow this reaction. ^{13}C CP-MAS NMR spectra showed clean conversion of $\mathbf{G^{HA}_{55}}$ to $\mathbf{Enl_{55}}$, with loss of alkene carbon resonances and formation new carbon resonances consistent with epoxide formation (Figure 1). Conversion was also monitored by FTIR, where the alkene C-H bend[23] at 992 cm^{-1} was replaced with a band consistent with an epoxide ring deformation[24] at 835 cm^{-1} (see Figure S1). Together, these data confirm the successful preparation of stable $\mathbf{Enl_{55}}$ with high conversion of alkene groups to epoxide functionality.

The solubility of $\mathbf{Enl_{55}}$ allowed for additional characterization of this polypeptide using gel permeation chromatography (GPC) and circular dichroism (CD) spectroscopy in HFIP solvent. The CD spectrum of $\mathbf{Enl_{55}}$ revealed that the chains primarily adopt α-helical conformations (Figure 2), similar to the precursor $\mathbf{G^{HA}_{55}}$.[11] The predominant α-helical conformation also indicates that significant racemization of the polypeptide backbone did not occur during the oxidation process. From literature on oxidation of homoallylglycine derivatives,[21,22] it was expected that both epoxide stereoisomers are formed

in approximately equal abundance during preparation of **Enl$_{55}$**. However, if this is the case the presence of different epoxide stereoisomers did not prevent **Enl$_{55}$** chains from adopting stable α-helical conformations. The GPC chromatogram of **Enl$_{55}$** showed a monomodal peak with dispersity of 1.26 (see Figure S2), commensurate with the dispersity of the **G$^{HA}_{55}$** precursor,[11] suggesting there was negligible chain degradation during the oxidation process. Overall, we found that **G$^{HA}_{55}$** could be converted to **Enl$_{55}$** in an efficient, straightforward process to give stable, soluble α-helical polypeptides containing epoxide side-chain functionality.

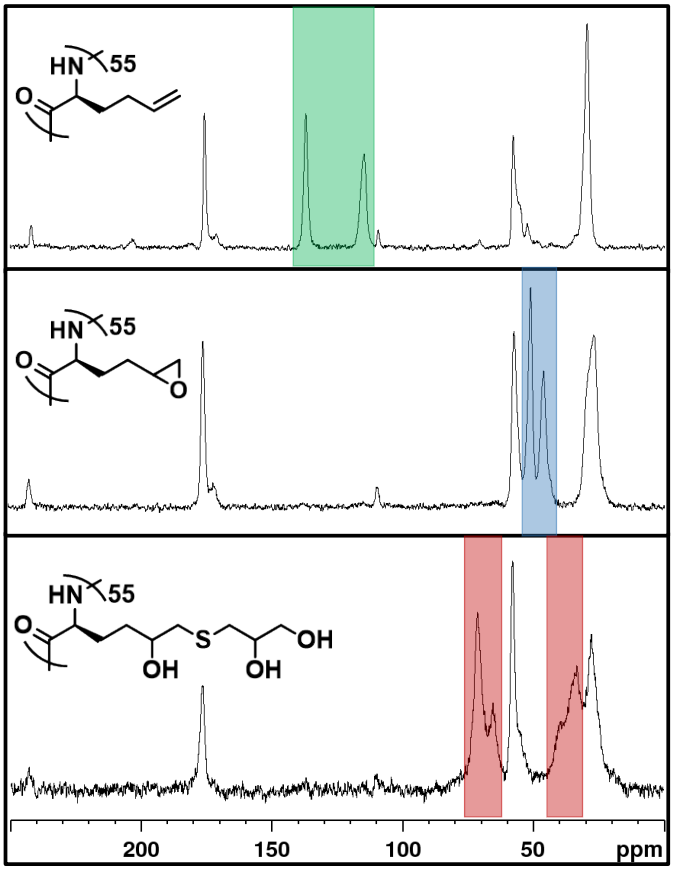


Figure 1. Stacked ^{13}C CP-MAS NMR spectra of A) **G$^{HA}_{55}$** B) **Enl$_{55}$** and C) **GL-Enl$_{55}$**. * = spinning sidebands. Green band highlights alkene carbons; blue band highlights epoxide carbons; red band highlights carbons of thiol addition product.

Epoxide containing polymers, notably poly(glycidyl methacrylate),[15,16] have been used extensively for post-polymerization modification reactions via conjugation with nucleophiles such as

thiols. Under basic conditions, the reactions with thiols proceed in high conversion and complete regioselectivity with thiols adding to the least hindered side of the epoxide groups.[15,16] To confirm that **Enl$_{55}$** can also serve as a precursor to functional polypeptides, it was reacted with a variety of water soluble thiols (Figure 3). In the presence of non-nucleophilic base 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), **Enl$_{55}$** was converted into eight different functional derivatives in high overall yields each with greater than 90% functionalization (Figure 3). Conversion of **Enl$_{55}$** to **R-Enl$_{55}$** derivatives was monitored using ^{1}H NMR, ^{13}C NMR, and FTIR, where resonances and bands for **Enl$_{55}$** were replaced with new resonances and bands for **R-Enl$_{55}$** that were consistent with the expected regiochemistry of addition (Figure 1, see Figure S1).[15,16,23] GPC analysis of **mEG$_4$-Enl$_{55}$** showed a monomodal peak that was shifted in retention volume compared to **Enl$_{55}$** and possessed similar dispersity as the precursor, which indicated negligible chain cleavage or crosslinking side-reactions (see Figure S2). These results confirmed that thiol addition to **Enl$_{55}$** is an efficient method for preparation of functional polypeptides.

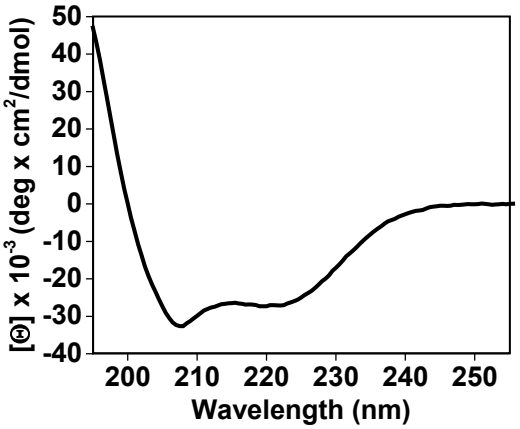
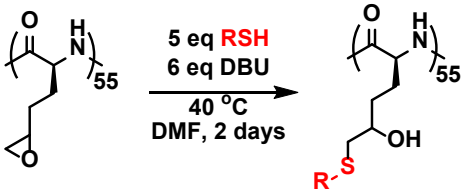


Figure 2. Circular dichroism spectrum of **Enl$_{55}$** in HFIP at 0.1 mg/mL, 20 °C.

Most of the resulting thioether containing **R-Enl$_{55}$** polypeptides were found to be soluble in DI water at 20 °C. **mEG$_1$-Enl$_{55}$** and **eEG$_2$-Enl$_{55}$** were not water soluble at concentrations as low as 0.1 mg/mL, likely due to the lower hydrophilicity of their side-chain groups. **CB-Enl$_{55}$** was also found to not dissolve in DI water, but was soluble in water at pH < 4, where the carboxylate groups are neutralized and this polypeptide becomes a polycation. CD spectra of all the water soluble, non-ionic oligoethylene glycol

(EG) functionalized **R-Enl$_{55}$** in DI water showed that these derivatives adopt α-helical conformations with calculated % helical contents ranging from *ca*. 48 to 63 % (Figure 4). Similar to other non-ionic polypeptides,[11,17] the α-helical content of **mEG$_2$-Enl$_{55}$** was found to increase when dissolved in methanol (see Figure S3), likely due to decreased solvation of EG groups in this solvent compared to water.[25] The conformation of non-ionic, thioglycerol functionalized **GL-Enl$_{55}$** was also found to be predominantly α-helical in DI water (see Figure S4), while charged **CB-Enl$_{55}$** at pH 2 was found to adopt a disordered conformation due to its polyelectrolyte nature (see Figure S5).



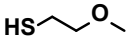
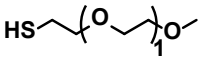
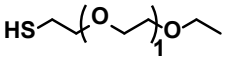
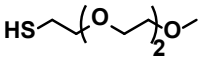
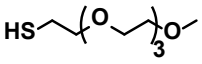
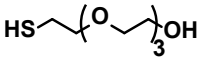
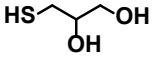
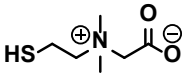
Sample	RSH	Funct (%)	Yield (%)
mEG$_1$-Enl		100	100
mEG$_2$-Enl		98	98
eEG$_2$-Enl		100	73
mEG$_3$-Enl		92	82
mEG$_4$-Enl		95	100
EG$_4$-Enl		100	88
GL-Enl		96	75
CB-Enl*		93	97

Figure 3. Base-catalyzed addition of thiols to **Enl$_{55}$**. Funct = mol percent functionalization of epoxide groups. Yield = total isolated yield of purified, functionalized polypeptide. * = reaction conditions: H_2O at 20 °C, 10 eq RSH per epoxide group.

While the water solubility and chain conformational preferences of **R-Enl$_{55}$** derivatives fit with expectations, these samples also provide insights into how subtle alterations of side-chain linkage chemistry affect these properties. For example, **R-Enl$_{55}$** derivatives are linkage isomers of **R-C^{H}**

polypeptides prepared via a different pathway (Scheme 2).[17] These two classes of functional polypeptides differ only in reversed orientation of side-chain thioether and pendent alcohol functional groups. The effect of this structural difference on solution chain conformation was examined by comparing aqueous CD spectra of **mEG$_2$-Enl$_{55}$** and "**mEG$_1$-C$^H_{60}$**" (from the literature),[17] which contain the same EG functionality and differ in residue composition by only a single methylene unit (Figure 5). In spite of this, a significantly greater α-helical content was observed for **mEG$_1$-C$^H_{60}$** (84% α-helix) versus **mEG$_2$-Enl$_{55}$** (50% α-helix), and is likely due to the hydrophilic side-chain hydroxyl groups being closer to the backbone in **mEG$_2$-Enl$_{55}$**, since solvation of these groups by water can enhance solvation of the backbone and promote α-helix destabilization.

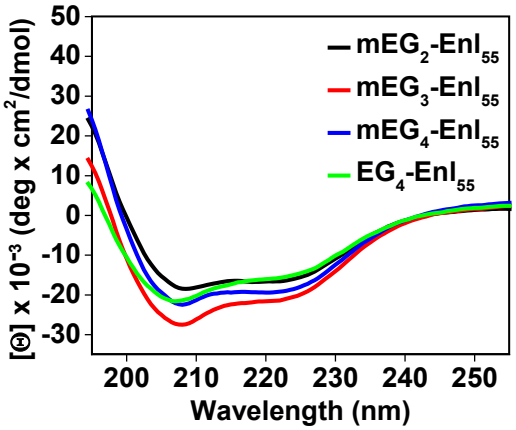


Figure 4. Circular dichroism spectra of **mEG$_2$-Enl$_{55}$** (black, 50% α-helix), **mEG$_3$-Enl$_{55}$** (red, 63% α-helix), **mEG$_4$-Enl$_{55}$** (blue, 57% α-helix), and **EG$_4$-Enl$_{55}$** (green, 48% α-helix) in DI water at 0.1 mg/mL, 20 °C.

The α-helix destabilizing effect of the hydroxyl groups in **R-Enl$_{55}$** was also observed in comparison of **mEG$_4$-Enl$_{55}$** with **mEG$_4$-G$^{HA}_{63}$** (from the literature)[11] in DI water (Figure 5). These polypeptides, both derived from a common precursor, **G^{HA}**,[11] differ only in the presence or absence of the side-chain hydroxyl groups. Increased lengths of EG segments were used in this comparison since they are required to obtain a water soluble **G^{HA}** derivative.[11] In this comparison, **mEG$_4$-G$^{HA}_{63}$** (71% α-helix)

was also found to possess a greater α-helical content versus **mEG$_4$-Enl$_{55}$** (57% α-helix), although the difference was not as substantial as in the comparison with **R-C$^{H}_{60}$**. As above, solvation of the hydroxyl groups of **mEG$_4$-Enl$_{55}$** in water is the likely reason for the lower α-helical content in this sample versus **mEG$_4$-G$^{HA}_{63}$**. Interestingly, the position of the side-chain thioether group also appears to have a significant effect on α-helix stability, as **mEG$_1$-C$^{H}_{60}$** possesses significantly greater α-helical content than **mEG$_4$-G$^{HA}_{63}$**. For a more meaningful comparison, we compared **mEG$_4$-G$^{HA}_{63}$** with a "**mEG$_4$-C$^{H}_{80}$**" polypeptide that contains the same side-chain, except that the sulfur atom is two carbons closer to the backbone (see Scheme S1).[18] The reported α-helical content of "**mEG$_4$-C$^{H}_{80}$**" is 98% in DI water,[18] confirming that the sample with sulfur atoms closer to the backbone forms a more stable α-helix in water, despite possessing overall shorter side-chains. These comparisons help to reveal how subtle structural differences can exert influence on polypeptide conformational tendencies.

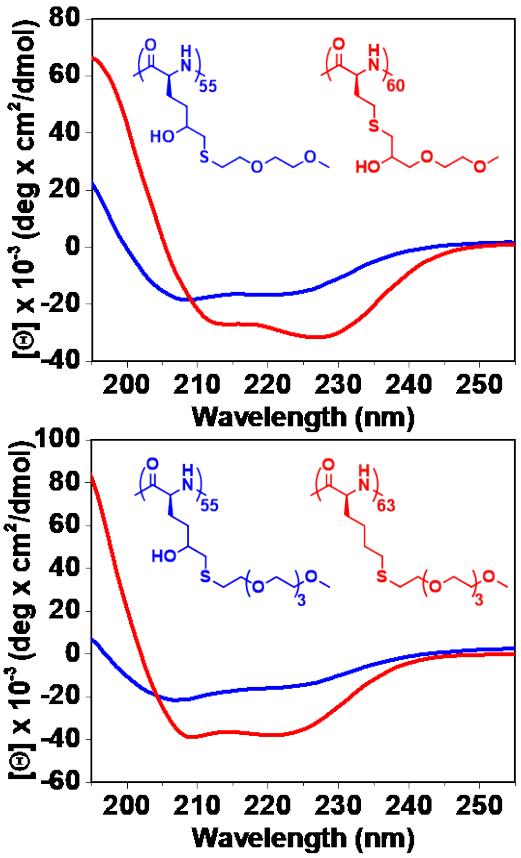


Figure 5. Comparisons between circular dichroism spectra of **R-Enl** derivatives and previously reported **R-C^{H}** and **R-G^{HA}** polypeptides**.** Circular dichroism spectra collected in DI water at 20 °C. A) **mEG$_2$-Enl$_{55}$** (blue, 0.1 mg/mL, 50% α-helix) and **mEG$_1$-C$^{H}_{60}$** (red, 0.5 mg/mL, 84% α-helix).[17] B) **mEG$_4$-Enl$_{55}$** (blue, 0.1 mg/mL, 57% α-helix) and **mEG$_4$-G$^{HA}_{63}$** (red, 0.5 mg/mL, 71% α-helix).[11]

Another characteristic feature of **EG$_n$-C^{H}** polypeptides is that they can possess temperature dependent water solubility, i.e. lower critical solution temperature (LCST) behavior, where they reversibly phase separate from solution at elevated temperature.[17] Aqueous solutions of the water soluble **R-Enl$_{55}$** polypeptides described above were heated to determine if these polymers also display temperature dependent solubility. Of these samples, only **mEG$_2$-Enl$_{55}$** underwent a reversible LCST transition with a cloud point temperature of 40 °C (Figure 6). The corresponding **EG$_n$-C^{H}** analog, **mEG$_1$-C$^{H}_{60}$** (*vide supra*), has a reported cloud point temperature of 33 °C in DI water.[17] The higher cloud point temperature of **mEG$_2$-Enl$_{55}$** suggests that these polypeptides with pendant hydroxyl groups closer to the chain backbone possess inherently greater water solubility compared to **EG$_n$-C^{H}** polypeptides.

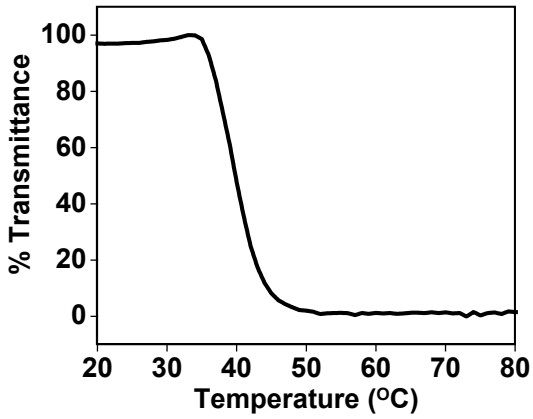
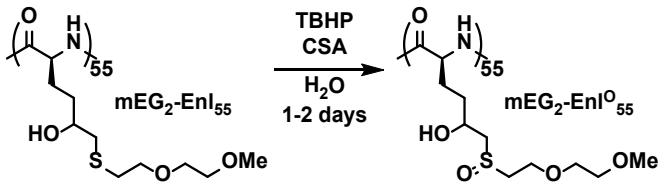


Figure 6. Thermoresponsive behavior of **mEG$_2$-Enl** at 10 mg/mL in DI water. Polypeptide solution shows a reversible LCST transition with a cloud point temperature of 40 °C.



Scheme 3. Oxidation of thioether containing polypeptide **mEG_2-Enl_{55}** to its corresponding sulfoxide derivative **mEG_2-Enl^O_{55}**.

In **EG_n-C^H** polypeptides, oxidation of thioether linkages to sulfoxides has been shown to result in an α-helix to coil conformational transition, as well as loss of LCST behavior in water.[17,18] The loss of thermoresponsive solubility is believed to be due to the increased hydrophilicity and water solvation of sulfoxide versus thioether groups. To extend the comparison between **mEG_2-Enl_{55}** and **mEG_1-C^H_{60}**, **mEG_2-Enl_{55}** was oxidized to its corresponding sulfoxide derivative **mEG_2-Enl^O_{55}**, which was obtained in high yield and possessed good water solubility (Scheme 3). The CD spectrum of **mEG_2-Enl^O_{55}** showed that it's α-helical content (*ca*. 23%) was much lower than the unoxidized precursor (*ca*. 50% α-helix), yet still retained some helical content (Figure 7). In contrast, the product of oxidation of **mEG_1-C^H_{60}**, i.e. **mEG_1-C^{HO}_{60}**, has been reported to be essentially completely disordered in DI water (*ca*. 5% α-helix) (Figure 7).[17] The difference in degree of conformational switching upon oxidation of **mEG_2-Enl_{55}** and **mEG_1-C^H_{60}** can be directly related to the position of the thioether groups in the polypeptide side-chains. The increased distance of sulfoxides from the backbone in **mEG_2-Enl^O_{55}** compared to **mEG_1-C^{HO}_{60}** results in a weaker influence of these groups on polypeptide backbone solvation, H-bonding, and α-helix stability, as has been observed in other related polypeptides.[11,17] Even though **mEG_2-Enl^O_{55}** did not become fully disordered upon oxidation, generation of hydrophilic sulfoxide groups was sufficient to increase chain solvation so that this polymer no longer possessed LCST behavior. Hence, **mEG_2-Enl_{55}** was found to be capable of redox switchable LCST behavior, similar to **EG_n-C^H** polypeptides.

Conclusions

A new epoxide containing polypeptide, **Enl**, was synthesized, characterized and found to be a readily prepared intermediate that can be reacted with thiols to give a variety of functional polypeptides. Since **Enl** chains are derived directly from $\mathbf{G}^{\mathbf{HA}}$ polypeptides, which can be prepared with controlled lengths and as discrete segments in block copolypeptides, it is conceivable that **Enl** can also be incorporated into more complex copolypeptide architectures. Beyond formation of functional conjugates with thiols, **Enl** was also useful for preparation of side-chain structural analogs of thioether containing polypeptides prepared via other routes. This feature allowed comparison of polypeptides containing similar functional groups, but with small structural differences in side-chain linkages that gave insights on how linkage structure influences polypeptide solubility, chain conformation and thermoresponsive behavior.

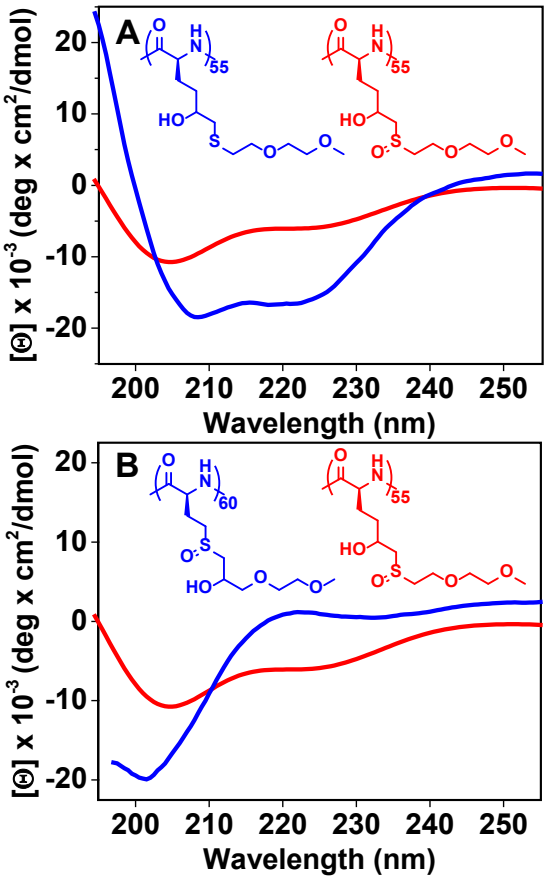


Figure 7. A) Comparison between circular dichroism spectra of **mEG$_2$-Enl$_{55}$** (blue, 50% α-helix) and **mEG$_2$-Enl$^{O}_{55}$** (red, 23% α-helix) showing change in chain conformation after oxidation. Both samples prepared at 0.1 mg/mL in DI water at 20 °C. B) Comparison between circular dichroism spectra of **mEG$_1$-C$^{HO}_{60}$** (blue, 0.25 mg/mL, 5% α-helix)[17] and **mEG$_2$-Enl$^{O}_{55}$** (red, 0.1 mg/mL, 23% α-helix). Both samples prepared in DI water at 20 °C.

Associated Content

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biomac.#######.

Additional figures, synthesis procedures, and spectral data for all new molecules (PDF).

Author Information

Corresponding Author

* demingt@seas.ucla.edu

ORCID

Timothy J. Deming: 0000-0002-0594-5025

Notes

The authors declare no competing financial interest.

Acknowledgments.

This work was supported by the National Science Foundation under grant CHE 1807362. The authors thank Dr. Robert E. Taylor (UCLA Chemistry and Biochemistry) for technical assistance with the solid-state ^{13}C CP-MAS NMR experiments.

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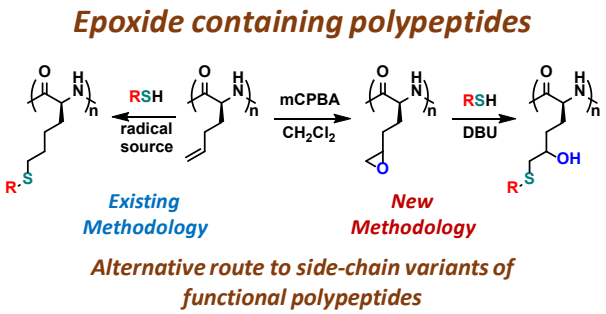
Epoxide containing polypeptides
RSH
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DBU
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Existing Methodology
New Methodology
Alternative route to side-chain variants of functional polypeptides